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A revised method for pigment extraction from marine nannoplanktonic algal cultures

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Abstract*:*
Three different extraction methods in four time periods were compared for the ability to extract Chl *a* and total carotenoids from marine nannoplanktonic alga, *Nannochloropsis salina*. Measurements were made with spectrophotometer using 90% acetone as the extraction solvent. Homogenized samples gave higher values of Chl *a* and carotenoids. Cell homogenization is essential for the release of pigments into the solvent and their accurate measurement, especially in nannoplanktons containing thick cell wall. A decrease in Chl *a* values were noted when the extracts were incubated which shows degradation of pigments during incubation. Addition of $MgCO_3$ as recommended in other methods did not prevent pigment degradation in the present study
Key words: Chlorophyll *a*, Total carotenoids, *Nannochloropsis salina*

Introduction*:*

Microalgae are microscopic unicellular organisms capable of converting solar energy to chemical energy via photosynthesis. They contain numerous bioactive compounds that can be harnessed for commercial use. Marine microalgae in particular have unique biochemical characteristics which are not found in higher plants (Rasmussen *et al*., 2007). Microalgae include both prokaryotic cyanobacteria and eukaryotic unicellular algae. The structural and functional simplicity of these microorganisms makes them a better choice for research purposes than any other terrestrial plants (Brennan and Owende, 2010). In addition, microalgae have rapid growth rates and higher productivities than any other plant systems. Microalgae can also grow in variable environmental conditions (Wang *et al*., 2008). Chl *a* is ubiquitous in microalgae, which constitute approximately 1 to 2 % of the dry weight of planktonic algae (APHA, 1985). They are extensively used as an indirect measure of biomass in both ecological and physiological studies of micro algae (Marker *et al*., 1980). Pigment analysis is also used as a method to study the trophic level of ecosystem. The quantification of Chl *a* is easier than the algal biomass itself (Dere *et al*., 1998; Gitelson *et al*., 2000) Photosynthetic efficiency and cell growth associated with quantification of Chl *a* under various conditions have been studied by Masjidek *et al*. (2000) and Tremblin *et al*. (2000). Presence or absence of the various photosynthetic pigments is used, among other features, to separate major algal classes (Buchaca *et al*., 2005; Llewellyn *et al*., 2005; Wuff *et al*., 2005). Carotenoid concentrations are often used to describe the composition of phytoplankton in water and to estimate their abundance in mixed populations (Llewellyn *et al*., 2005; Wulff *et al*., 2005; Schagerl and Kunzl, 2007). However, estimation of the ratio of carotenoids to Chl *a* is useful to understand the action of organism to changing environmental light condition (Jodłowska and Latała, 2010). Spectrophotometry, Fluorometry and High Performance Liquid Chromatography (HPLC) are extensively used for the qualitative and quantitative estimation of pigments.

Spectrophotometric analysis is considered as one of the reliable, fast, simple and cost effective method for algal pigment analysis (Sabina and Adam, 2011). Routine spectrophotometric methods are not able to distinguish between Chl-ide, phaeophorbide and chlorophyll (Marker, 1972) despite this flaw, spectrophotometry is still the most widely used method for measuring chlorophyll in aquatic system because it is fast and simple (Otsuki and Takamura, 1988; Pepe *et al*., 2001).

Louda and Monghkonsri (2006) compared spectrophotometric estimates of chlorophyll contents with those obtained by High Performance Liquid Chromatography (HPLC) and concluded that spectrometric evaluation of chlorophyll, using UNESCO (1966) Jeffrey and Humphry (1975) equations gave excellent results.

Spectrophotometric analysis is less expensive and much faster than HPLC analysis, making them a good tool for routine chlorophyll estimation (Henriques *et al*., 2007). The results of chlorophyll and carotenoids concentration obtained by HPLC analysis were compared with the results from spectrophotometer. High values of correlation

coefficients (r = 0.970 for chlorophyll, 0.978 for carotenoid and 0.997 for Car./Chl.) show a great concurrence of the results obtained by two independent methods (Sabina and Adam, 2011).

Nannochloropis salina used in the present study as an experimental organism is a marine unicellular nannoplankton belonging to the family Eustigmatophyceae having only Chl *a*. *Nannochloropsis* is a promising biological tool for the standardization of pigment estimation (Henriques *et al*., 2007).

Selection of solvent to promote the extraction is a very important issue since it determines the degree of affinity of the solvent to the chemical substances to be extracted. Apart from dissolution ability towards the compounds to be extracted and quantified, the solvent also plays an important role in cell lysis. More aggressive solvents can increase the extraction yield in cells with strong walls. Methanol was the first solvent to be used to extract chlorophylls, but due to its toxicity it has been replaced by other. Different solvents are used in algal pigment extraction but the most common solvent is acetone (Havskum *et al.,* 2004; Buchaca *et al.,* 2005). Acetone is not only superior in chlorophyll extraction; it also caused less artifacts than the other solvents (Schager and Kunzl, 2007). Grinding in acetone obtained better extraction (Wright *et al.*, 1997; Gerloff- Elias *et al.*, 2005). Extraction efficiency may relay on cell concentration, Chl *a* content, type of extraction of solvent, extraction time and species of algae used (Rai, 1980). A perfect extraction procedure required fast and reproducible results (strain *et al.*, 1971; Zapata and Garrido, 1991). Though different methods are available, a reliable method for pigment extraction from nannoplanktons with thick cell walls is lacking. Hence the present study aims to standardize pigment extraction procedure for the spectrophotometric quantification of chlorophyll and carotenoids from marine nannoplanktonic algal cultures.

Materials and Methods:

Strain and culture conditions: *Nannochloropsis salina* is a unicellular alga included in the family Eustigmatophyceae with thick cellulose cell wall. *N. salina* culture maintained in the Marine Botany laboratory was used for the study. Cultures were raised in 500 ml Erlenmeyer flasks, containing 300ml f/2 - Si medium (Guillard, 1975) of salinity 30 ppt. Illumination was provided by cold white fluorescent light of 2000 lux for a light/ dark period 12:12 hours. Cultures were maintained at room temperature (30 ± 2 ^{0}C). The cell concentration of microalga was evaluated by reading the absorbance at 690 nm in a Hitachi U- 2001 spectrophotometer. The absorbance at 690 nm was correlated to cell dry weight. Cell counting was done using Heamocytometer. Growth kinetics of the cultures was studied and cultures in exponential phase were used for pigment extraction.

Sample preparation: A volume of 1 ml culture sample in Log phase was withdrawn. The culture was filtered through 13 mm Whatman GF/C filters (gentle vacuum filtration).

Extraction Method: The filters with algal cells were introduced in to a screw capped test tube containing 5 mL of 90% (v/v) acetone. The test tube was covered with aluminium foil to prevent the entry of light. Selected samples were grinded in a glass homogenizer and refrigerated for different time intervals; 1, 6, 12 and 24 hours. The samples after incubation period were centrifuged at 3000 rpm for 5 minutes. The clear supernatant was taken, made up to 5 m L with 90% acetone and used for pigment quantification. Pigment extraction was done in three methods. In the first method, algal sample was homogenized with a glass homogenizer. In the second method non homogenized sample was used for pigment extraction, whereas in the third method sample was homogenized with the addition of 0.2 ml, 1% $MgCO_3$ as a preservative. All these experiments were done in triplicate and performed at dark.

Quantification: Absorbance of the sample was noted using 90% acetone as blank, at 750, 665, 645, 630 and 480 nm in Hitachi U 2001 spectrophotometer. Subtracted the extinction at 750nm from the extinctions at 665, 645, 630 and 480 nm (Jeffrey and Humphry,1975). The amount of Chl *a* in the sample was estimated using the equation.

Ca = 11.85 (OD 665) - 1.54(OD 645) - 0.08 (OD 630) (Jeffrey and Humphrey, 1975)

Chlorophyll *a* µg/L = Ca X Extract volume, mL / Volume of sample, L

C car = 4 (OD 480) (Strickland and Parsons, 1972).

Total carotenoids µg/L = C car X extract volume/ Volume of sample, L

Statistical analysis: Statistical analysis was done with SPSS version 11, one way analysis of variance (ANOVA) was done for values of Chl *a* and carotenoids. Higher values of Chl *a* and carotenoids in all the three methods were compared using Tukeys Test ($p< 0.05$).

Results*:*

The chlorophyll and carotenoids values obtained from the first method (homogenized sample) after one hour extraction were 3217± 525 µg/L and 1280 ± 72 µg/L respectively whereas in the second method where non homogenized samples were used the values were, Chl 450.2 ± 198.4 µg/L and carotenoids, 753.3 ± 110.2 µg/L. Third method yielded 2012± 701 µg/L Chl and 1013± 122 µg/L carotenoids after one hour incubation. (Fig.1 and Fig. 2).

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Higher chlorophyll values were obtained when the algal cells were homogenized using mechanical disruption methods compared to samples without disruption ($r = 0.001$, $p < 0.05$).

There was a reduction in pigment values during incubation. Chlorophyll values showed a reduction from 450.2 ± 198.4 µg/L to 184 ± 32 µg/L and total carotenoids from 753.3 ± 110.2 µg/L to 573.3± 11.5 µg/L when non homogenized samples were kept for 24 hours. In homogenized samples the Chl value reached from 3217 ± 255 µg/L to1806.7 ± 543.4 µg/L. However there was a slight increase in carotenoid value (from 1013.3 ± 180 µg/L to 1280 ± 72 µg/L), after 24 hours incubation. A significant decrease in pigment quantity was noticed when samples were incubated (Chl: $r = 0.015$, $p< 0.05$) (Car: $r = 0.076$, $p< 0.05$). The Chl and carotenoid values obtained after the addition of Mg CO_3 was also significantly less compared to samples without $MgCO_3$ (Chl *a* $r = 0.037$, $p< 0.05$) (Car: $r = 0.045$, $p< 0.05$).

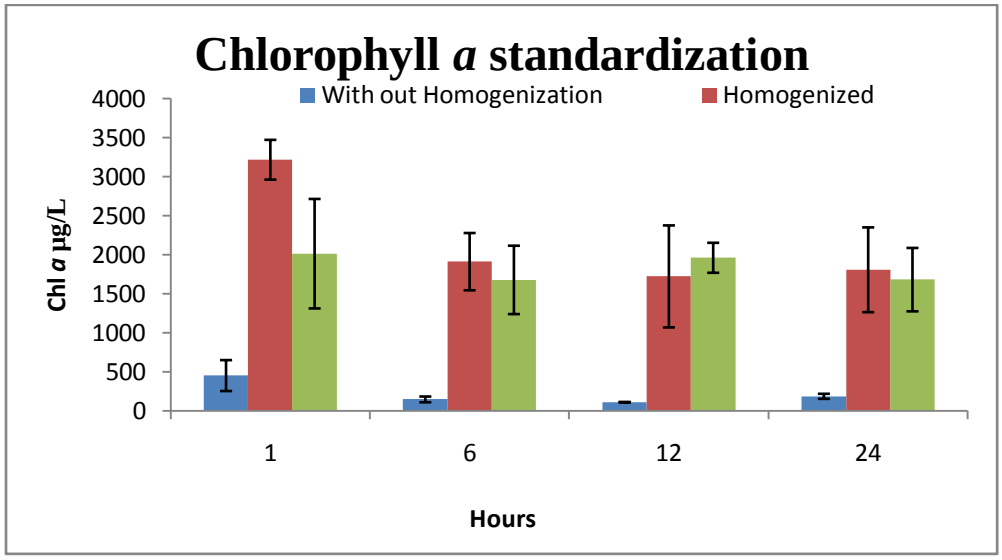

Fig 1: Chl *a* of *N. salina* obtained by spectrophotometry

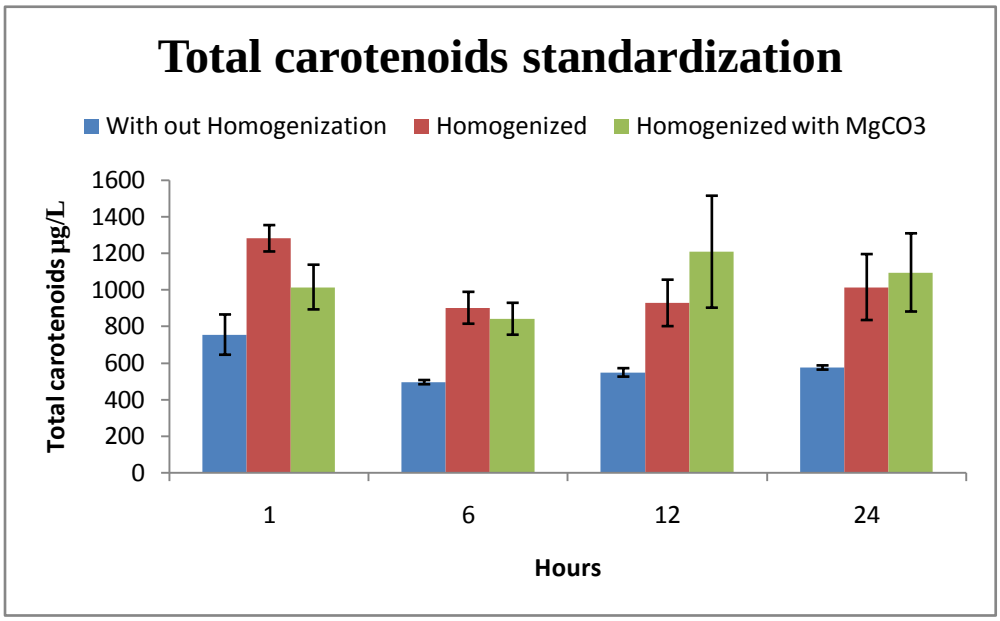

Fig 2: Total carotenoids of *N. salina* obtained by spectrophotometry

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Discussion*:*

Chlorophyll estimation is extensively used as an indirect measure of biomass both in ecological and culture studies related to algae (Marker *et al*., 1980). Suitable extraction procedures are one of the key steps in pigment analysis (Rai, 1980). Different pigment extraction methods are used now a days but a majority of them do not yield good extraction of pigments from the cells.

In the present study, three different methods of pigment extraction was used to optimize the extraction technique. Better results in chlorophyll values were obtained when the algal cells were homogenized using mechanical disruption methods compared to samples without disruption ($r = 0.001$, $p < 0.005$). Pigment analysis with homogenization produced definitely higher amount of Chl *a* and total carotenoids. In many methods including Parson *et al*. (1984) pigment estimation is described without giving importance to cell homogenization. Analysis without homogenization produced lower Chl concentrations of around 50% compared to homogenized samples (Schagerl and Kunzl, 2007). Many workers use sonicators for the homogenization of samples (Michael and Gabriela, 2007). Handling of the sonicator was more complicated than homogenization and this disadvantage was paired with lower extraction efficiency. The vibrations of the probe causes air bubbles and extensive production of foam and heat in the extract (Schagerl and Kunzl, 2007). Chlorophyll and Carotenoids are easily degraded by heat (Braumann and Grimme, 1979). The period of extraction also affects the efficiency of pigment extraction. In the present study, a decrease in chlorophyll value was observed when the period of extraction was 24 hours. The Chl *a* value reached from 450.2 ± 198.4 µg/L to 184 ± 32 µg/L and total carotenoids from 753.3 ± 110.2 µg/L to 573.3± 11.5 µg/L, when non homogenized samples were kept for 24 hours. In homogenized samples also the chl *a* value was reduced from 3217 ± 255 µg/L to1806.7 ± 543.4 µg/L after 24 hours incubation. The extraction period is one of the major time consuming part of pigment estimation. The period of extraction suggested was 15- 24 hours in several methods (Strickland and Parsons, 1972; Shenbaga *et al*., 2012) but in our study the optimum extraction period observed was one hour.

Addition of Mg CO_3 is an established practice during pigment extraction. But in the present study addition of $MgCO_3$ did not increase the yield of pigments, but reduced the Chl value. The sample with homogenization and addition of Mg CO_3 gave a value of 2012 ± 701 µg/L for Chl *a* which was around 37% lesser than the samples without $MgCO_3$ (3217 ± 525 µg/L, Chl *a*). Carotenoids values obtained after the addition of Mg CO_3 was also significantly less compared to sample without $MgCO_3$ (Chl: $r = 0.037$, $p< 0.05$) (Car: $r = 0.045$, $p< 0.05$). The study shows that MgCO3 cannot prevent chlorophyll degradation. Strickland and Parsons (1972) also doubted the efficacy of such an addition.

Sample size is also an important factor during pigment extraction. In the present study, only 1 mL sample was taken for pigment extraction; this small amount of sample will not affect the growth of the algal culture (Kumar and Saramma, 2012). But in most of the studies, sample volume of 10 mL is advised (Shenbaga *et al*., 2012). Large sample size will adversely affect small scale batch culture. A small sample size will not affect the culture setup and it will minimize the errors arising from spectrophotometric reading. The volume of solvent used for extraction was only 5 mL rather than 10ml recommended in other studies (Strickland and Parsons,1972; Becker, 1994) which reduces the cost of the assay.

The present study shows that homogenization of the cells without addition of $MgCO_3$ is an easy, low cost, reliable and precise method for the extraction and spectrophotometric measurement of pigments in thick walled microalgae such as *Nannochloropsis salina*

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